Intrathymic administration of hematopoietic progenitor cells enhances T cell reconstitution in ZAP-70 severe combined immunodeficiency

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Edited by Leonard A. Herzenberg, Stanford University School of Medicine, Stanford, CA, and approved August 2, 2005 (received for review May 23, 2005)

Patients with severe combined immunodeficiency (SCID) present with opportunistic infections that are almost universally fatal in infancy. The mainstay treatment for these patients is allogeneic hematopoietic stem cell (HSC) transplantation, but sustained polyclonal T cell reconstitution is too often unsatisfactory. Although transplantation is conventionally performed by i.v. administration of HSC, we hypothesized that an intrathymic strategy would be superior. Indeed, several progenitor cell populations are incapable of homing to the thymus, the major site of T cell differentiation, and it appears that there are extensive time periods during which the thymus is refractory to progenitor cell import. To test this hypothesis, nonconditioned infant ZAP-70-deficient SCID mice were intrathymically injected with WT bone marrow progenitor cells, a procedure accomplished without surgical intervention. Upon intrathymic HSC injection, there was a more rapid T cell differentiation, with mature thymocytes detected by 4 weeks after transplantation. Intrathymic injection of HSC also resulted in significantly higher numbers of peripheral T cells, increased percentages of naïve T cells, and more diverse T cell receptor repertoires. Moreover, T cell reconstitution after intrathymic transplantation was obtained after injection of 10-fold fewer donor HSC. Thus, this intrathymic transplantation approach may improve the outcome of SCID patients by enhancing T cell reconstitution.

stem cell transplantation | homing | thymus | T cell receptor repertoire

Patients with severe combined immunodeficiencies (SCID) have opportunistic infections that are almost universally fatal in infancy. Transplantation of histocompatible hematopoietic stem cells (HSC) from a related donor is the optimal treatment for infants with this clinical condition. In the absence of histocompatible donors, SCID patients typically receive an HSC transplant from HLA-haploidentical donors. In the latter situation, T cells are extensively depleted from the graft in an effort to prevent graft versus host disease. Although recent modifications of this protocol have resulted in an increased survival rate approaching 75%, significant short-term and long-term complications are still reported (1). The success of HSC treatment is also coupled to the kinetics of T cell reconstitution. Indeed, the emergence of significant numbers of circulating naïve T cells, capable of immune responsiveness, often requires >150 days, irrespective of whether the patient is transplanted with HLA-identical or -haploidentical HSC (2, 3). Thus, although this treatment is generally extremely successful, the lag time during which the patient continues to be susceptible to infections is quite long.

The generation of T cells from donor HSC is generally thought to occur in the thymus. Thus, donor HSC, administered by i.v. injection, must home to the thymus before their differentiation. In humans, it is not clear if these i.v.-injected cells, whether true HSC or progenitors with T lineage potential, first home to the bone marrow (BM) and are then exported to the thymus or, alternatively, directly enter the thymus. At least in mice, it is known that the latter scenario exists. Specifically, Spangrude and colleagues (4) showed that i.v.-injected BM-derived progenitor cells enter the thymus within 4 h after infusion. Nevertheless, it appears that the murine thymus is not continually receptive to the import of hematopoietic progenitors, alternating between refractory and responsive periods (5). During these refractory periods, donor progenitor cells differentiate into T cells only if they are directly injected into the thymus (5). Thus, although HSC transplantation is performed by an i.v. injection of cells into the peripheral circulation, T cell reconstitution of SCID patients might be enhanced if the HSC/progenitor cells are injected directly into the thymus.

This hypothesis was tested in a murine model of ZAP-70 deficiency. ZAP-70 is a 70-kDa protein tyrosine kinase that is recruited to the T cell receptor (TCR) after receptor stimulation (6). It is expressed at approximately equivalent levels in thymocytes, mature T cells, and natural killer cells (7), and its absence results in a SCID phenotype with a block in T cell development at the $CD4^+CD8^+$ thymocyte stage (8–10). Here, we demonstrate that direct intrathymic (IT) injection of allogeneic stem/progenitor cells into ZAP-$70^{-/-}$ mice results in a more rapid T cell differentiation than that observed after their i.v. injection, and the TCR repertoire of the generated T cells is significantly more diverse. Moreover, 10-fold fewer donor cells are required to generate mature peripheral T cells upon IT injection. Unexpectedly, we also found that in the absence of any myeloablative treatment, thymic differentiation was sustained for a longer time period after direct IT injection of HSC.

Methods

Bone Marrow Transplantation Protocols. ZAP-$70^{-/-}$ mice, kindly provided by A. Singer and R. Bosselut (National Institutes of Health, Bethesda), were bred and maintained under pathogen-free conditions. Donor BM cells, harvested from femurs and tibias of WT C57BL/6J or 129/SvPas mice, were first incubated with a mixture of specific Abs directed against lineage markers (TER119, B220, MAC-1, GR-1, and CD4, all rat α mouse hybridoma Abs) and then with α-rat IgG magnetic beads (Dynal, Compiegne, France) to remove differentiated hematopoietic cells. These lineage-negative (lin-) progenitor cells (2×10^3 to

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: BM, bone marrow; BMT, transplantation; HSC, hematopoietic stem cell; IT, intrathymic ; lin-, lineage-negative; SCID, severe combined immunodeficiency; SP, single-positive; TCR, T cell receptor; TCRBV, TCRβ chain hypervariable region.

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5×10^5 as indicated) were either injected i.v. into the tail vein (50 μl of volume) or directly into the thymus (25 μl of total volume) by insertion of a 0.3-ml 28-gauge 8-mm insulin syringe through the skin into the thoracic cavity immediately above the sternum. Mice were anesthetized with isoflurane before the latter procedure.

Immunophenotyping and Flow Cytometry Analyses. Cells, isolated from lymph nodes, spleen, and thymus, were stained with the appropriate conjugated αCD3, αCD25, αCD69, αCD62L (Immunotech), αCD4, αCD8, and αCD44 (Pharmingen) mouse mAbs, as indicated. Stained cells were analyzed by flow cytometry (FACSCalibur, Becton Dickinson).

T Cell Repertoire Analysis. Total RNA samples were prepared from splenocytes by using RNAlater (Ambion, Austin, TX) at 12–14 weeks after transplantation. RNA was reverse transcribed with random hexanucleotides (Amersham Pharmacia Biotech) by using Moloney MLV reverse transcriptase (GIBCO). cDNAs were amplified with one of 22 TCRβ chain hypervariable region (TCRBV) subfamily specific primers (5.3 and 19 subfamilies were excluded because of their poor amplification from control WT samples) and a Cβ primer recognizing the two constant regions Cβ1 and Cβ2 of the β chain of the TCR, as described in refs. 11 and 12). The TCRBV/Cβ-first run PCR products were subjected to elongation by using a Cβ dye labeled (6-Fam) primer allowing PCR products to be detected on a 337 automated DNA sequencer (Applied Biosystems). PCR products were analyzed for size and fluorescence intensity by using the Immunoscope software.

Statistical Analyses. Statistical significance was determined by using a Student's t test with a one-tailed distribution and two-sample equal variance. Data were considered to be statistically different for $P \leq 0.05$. All data are presented as means $\pm$ SD.

Results

Intrathymic Injection of Wild-Type BM Cells Results in Enhanced T Cell Reconstitution in ZAP-70$^{-/-}$ Mice. To determine the ability of IT-injected BM cells to reconstitute the T cell compartment in a SCID condition, we compared T lymphocyte differentiation after i.v. and IT administration of BM progenitor cells (2–5 $\times$ 10^5 lin- BM cells) in 2- to 3-week-old infant ZAP-70$^{-/-}$ mice. Mice were killed at 12–14 weeks after transplantation, and both CD4$^+$ and CD8$^+$ T cells were detected in the spleens and lymph nodes of the vast majority of transplanted animals (>80%; Fig. 1*A*). Importantly though, T cell reconstitution was significantly higher when BM cells were administered by IT injection (means of 75 $\times$ 10^6 and 26 $\times$ 10^6 splenic T cells in IT vs. i.v.-injected mice, $P = 0.0008$; Fig. 1*B*). As expected from these data, there was also a significantly higher overall percentage of peripheral T cells in IT-reconstituted animals (data not shown). Thus, IT administration of lin- BM progenitor cells results in enhanced T cell reconstitution as compared with conventional i.v. transplantation.

At early time points after HSC transplantation, or when T cell differentiation is limiting, there are only few T cells in the periphery. Under these conditions of lymphopenia, peripheral T cells undergo homeostatic proliferation in the absence of antigenic stimulation, with a transition from a naive to memory phenotype and the acquisition of activation markers. It was therefore important to compare the activation status of T cells in WT mice with ZAP-70$^{-/-}$ mice reconstituted by i.v. and IT administration of lin- BM cells. Indeed, the percentages of T cells expressing the CD69 activation marker were higher in reconstituted mice than in WT mice (means of 43% and 26% in i.v. and IT reconstituted ZAP-70$^{-/-}$ mice, respectively, vs. 9% in WT mice). This result correlated with lower percentages of naive T cells (as measured by expression of CD62L), strongly suggestive of a homeostatic proliferation in ZAP-70$^{-/-}$ mice reconstituted by administration of WT BM cells. Notably though, the percentage of naive CD62L$^+$ T lymphocytes was significantly higher when BM cells were administered by IT injection as compared with i.v. injection (mean of 54% vs. 25%, $P = 0.003$), and the percentage of memory/effector T cells (CD44^{hi}) was correspondingly lower (Fig. 2). Thus, peripheral T cell reconstitution in ZAP-70$^{-/-}$ mice is significantly improved, with a higher number of naive T cells, under conditions where lin- BM progenitor cells are administered by direct IT injection.

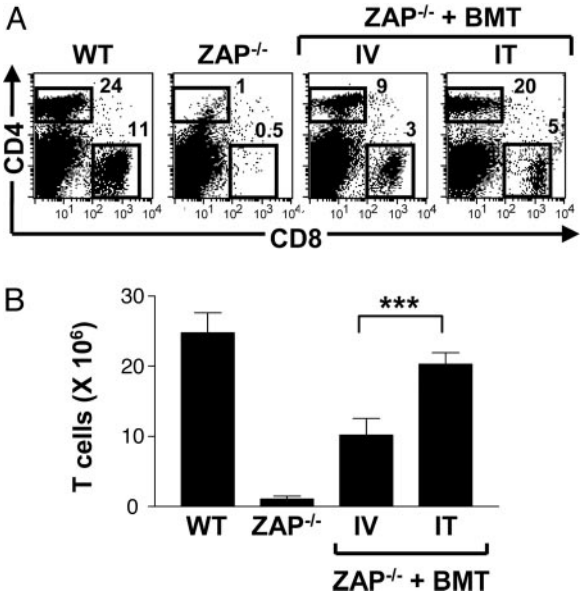

Fig. 1. T cell reconstitution in ZAP-70$^{-/-}$ mice after i.v. and IT administration of WT BM progenitor cells. (*A*) ZAP$^{-/-}$ mice were injected with lin- WT BM progenitor cells (2–5 $\times$ 10^5 per mouse) by either i.v. or IT routes (BMT). The presence of splenic T cells was monitored at 13 weeks after BMT by using PE-conjugated α-CD8 and Cy-conjugated α-CD4 antibodies. The percentages of CD8$^+$ and CD4$^+$ T cells from these representative WT, ZAP-70$^{-/-}$, and reconstituted mice are indicated. (*B*) Bar graph quantification of CD3$^+$ T cells in ZAP-70$^{-/-}$ mice injected with WT HSC at 3 weeks of age. Results are presented as means $\pm$ SD ($n = 4$). ***, $P = 0.0008$.

In the absence of the WT ZAP-70 protein, early and late biological responses, such as proliferation, are defective (8–10). As such, the peripheral T cells developing in both i.v.- and IT-reconstituted ZAP-70$^{-/-}$ mice were assessed for their ability to respond to T cell receptor engagement. In the absence of stimulation, neither T cells from WT mice nor IT-reconstituted mice proliferated. There was a low level of spontaneous proliferation of peripheral T cells isolated from i.v.-reconstituted mice (>5%), likely due to the more activated state of these lymphocytes (see above). Importantly, after a 3-day stimulation through CD3 and the CD28 coreceptor, the vast majority of T cells from both i.v.- and IT-reconstituted mice divided. Again though, T cells from i.v.-reconstituted mice divided more robustly than WT T cells, whereas the division profile of T cells from IT-reconstituted mice was similar to that of T cells from WT mice (Fig. 3*A*). Altogether, these data show that mature T lymphocytes differentiating after either i.v. or IT transplantation of HSC in ZAP-70$^{-/-}$ mice are capable of responding to TCR stimulation.

Increased T Cell Diversity After Transplantation of Progenitor Cells by Direct Intrathymic Injection. In humans, it has been shown that the T cell repertoire is severely skewed or oligoclonal until >100 days after bone marrow transplantation (BMT) (13). Thus, we assessed whether the peripheral T lymphocytes in the i.v. and IT ZAP-70-reconstituted mice were polyclonal or, alternatively,

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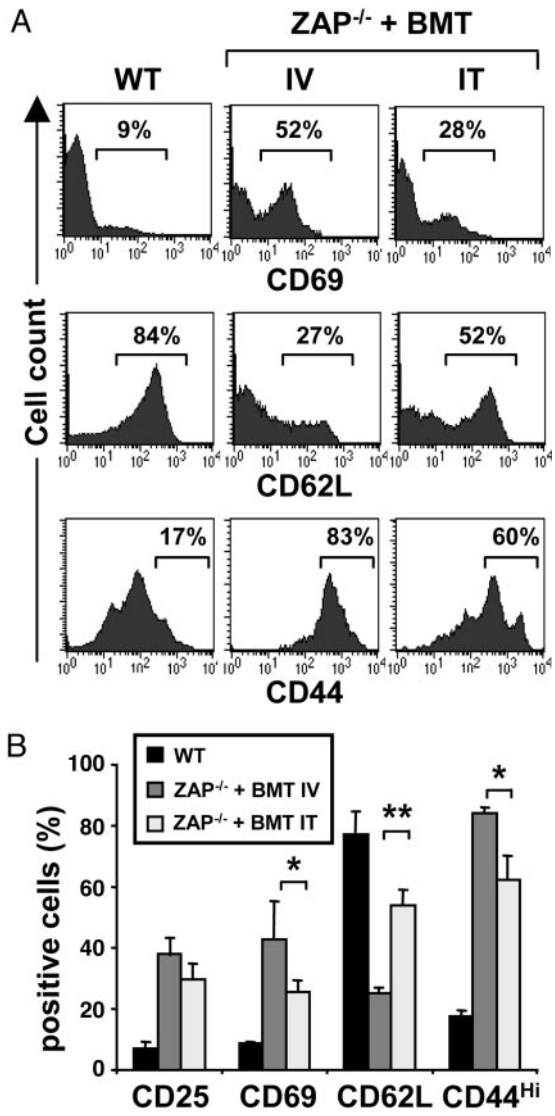

Fig. 2. T cell phenotype in ZAP-70-deficient mice reconstituted by i.v. and IT administration of WT BM progenitor cells. ZAP$^{-/-}$ mice were injected i.v. or IT with WT lin- BM cells (2×10^5 cells) at 3 weeks of age. Lymph node cells were analyzed at 14 weeks after BMT. (*A*) The phenotype of CD3$^+$ T cells was determined by using α-CD69, α-CD62L, and α-CD44 antibodies. (*B*) Bar graph quantification of CD3+ T cells positive for CD25, CD69, CD62L, and CD44 markers are presented as means $\pm$ SD ($n = 3$ for all groups). *, $P \leq 0.05$; **, $P \leq 0.01$.

arose from the expansion of a clonal or oligoclonal population of T cells.

Polyclonality was investigated at 12–14 weeks after transplantation by using the Immunoscope/spectratype method that determines the relative usage of each TCRBV within the global T cell population (11, 12). Representative panels showing Immunoscope results of PCR-amplified products from selected individual TCRBV families are shown in Fig. 3. As expected, the distribution of peaks in the vast majority of the BV families from WT mice showed a Gaussian distribution, indicative of a diverse and nonbiased T cell population. Transplantation of WT BM cells, by either i.v. or IT administration, resulted in the detection of repertoires representing most BV families. Nevertheless, animals reconstituted by i.v. administration of BM cells ($n = 7$) demonstrated a significantly less diverse TCR repertoire with polyclonal Gaussian profiles in only 12 $\pm$ 11% of families as compared with IT-reconstituted animals ($n = 5$) where 34 $\pm$ 18% of families were polyclonal ($P \leq 0.01$; Fig. 3 *B* and *C*). The ensemble of these data indicates that under conditions where lin- BM cells are administered by IT injection, there is an increased T cell receptor diversity after transplantation.

Distinct Kinetics of Thymocyte Differentiation in ZAP-70$^{-/-}$ Mice After i.v. and Intrathymic Injections of BM Progenitor Cells. The absence of ZAP-70 is associated with a relatively late block in T cell differentiation, at the CD4$^+$CD8$^+$ double-positive thymocyte stage (8–10) (Fig. 4). Because one of the problems in transplanted SCID patients is the length of time necessary to reconstitute the T cell compartment, it was important to determine the kinetics of thymocyte differentiation after i.v. and IT reconstitution. It has been shown that generation of single-positive (SP) mature thymocytes from immature thymocytes generally requires at least 26–28 days (14, 15). Notably, at 4 weeks after transplantation, SP thymocytes were detected in all IT-injected animals and none of the i.v.-treated mice (Fig. 4). Nevertheless, by 8 weeks after BMT, SP thymocyte differentiation was also observed in i.v.-injected animals (Fig. 4). Thus, it appears that under conditions where T precursor cells are "deposited" directly in the thymus, rather than into the peripheral circulation, their differentiation into mature thymocytes occurs with more rapid kinetics. Moreover, thymopoiesis in IT-reconstituted mice was sustained for a longer time period, with SP thymocytes detected at 13–14 weeks after the injection of lin- WT BM cells in seven of eight transplanted ZAP-70$^{-/-}$ mice, albeit with significantly higher numbers of CD4 SP cells. This finding is in contrast with i.v.-reconstituted ZAP-70$^{-/-}$ mice, where neither CD4 nor CD8 SP thymocytes were detected in 9 of 11 mice killed at 14 weeks after transplantation (Fig. 4). Altogether, the ensemble of these data shows a more robust thymocyte differentiation in ZAP-70$^{-/-}$ mice reconstituted by IT administration of HSC.

Transplantation of Limiting Numbers of BM Progenitor Cells Results in T Cell Reconstitution in IT- but Not i.v.-Injected ZAP-70$^{-/-}$ Mice. In the experiments presented in Fig. 1, T cell reconstitution was detected after both i.v. and IT administration of lin- BM cells. Nevertheless, in those experiments, the numbers of progenitor cells were high, 1×10^7 lin- progenitor cells/kg (2×10^5 per mouse). To assess the efficacy of T cell reconstitution after administration of more limiting numbers of progenitor cells, ZAP-70$^{-/-}$ mice were transplanted with either 2×10^3 or 2×10^4 lin- cells, administered by i.v. or IT routes. None of the mice transplanted with the lowest dose of cells, 2×10^3, presented with peripheral T cells at 14 weeks after transplantation (defined as >3% CD3$^+$ cells; $n = 6$; Fig. 5*A*). However, at a dose of 2×10^4 lin- cells, IT-treated animals developed T cells, whereas none of the i.v.-injected animals demonstrated T cell reconstitution. As expected, all animals transplanted with a 10-fold higher dose of cells, i.e., 2×10^5 cells, had peripheral T lymphocytes (Fig. 5*A*). Thus, at a limiting dose of lin- cells, T cell reconstitution was significantly boosted by directly injecting the cells into the thymus.

Notably though, there were lower numbers of peripheral T lymphocytes after the IT injection of 2×10^4 lin- cells as compared with 2×10^5 lin- cells. Because the presence of lower numbers of peripheral T cells can lead to lymphopenia-induced homeostatic proliferation, we monitored expression of activation markers on T cells from these IT reconstituted animals. Indeed, the percentages of T lymphocytes expressing the activation marker CD69 and the memory marker CD44 were significantly higher in ZAP-70$^{-/-}$ mice transplanted with 2×10^4 lin- cells ($P \leq 0.01$), whereas expression of the naïve marker CD62L was significantly lower ($P = 0.002$) (Fig. 5*B*). Interestingly, the phenotype of the peripheral T cells analyzed in the ZAP-70$^{-/-}$ mice receiving 2×10^4 BM cells through IT injection was similar

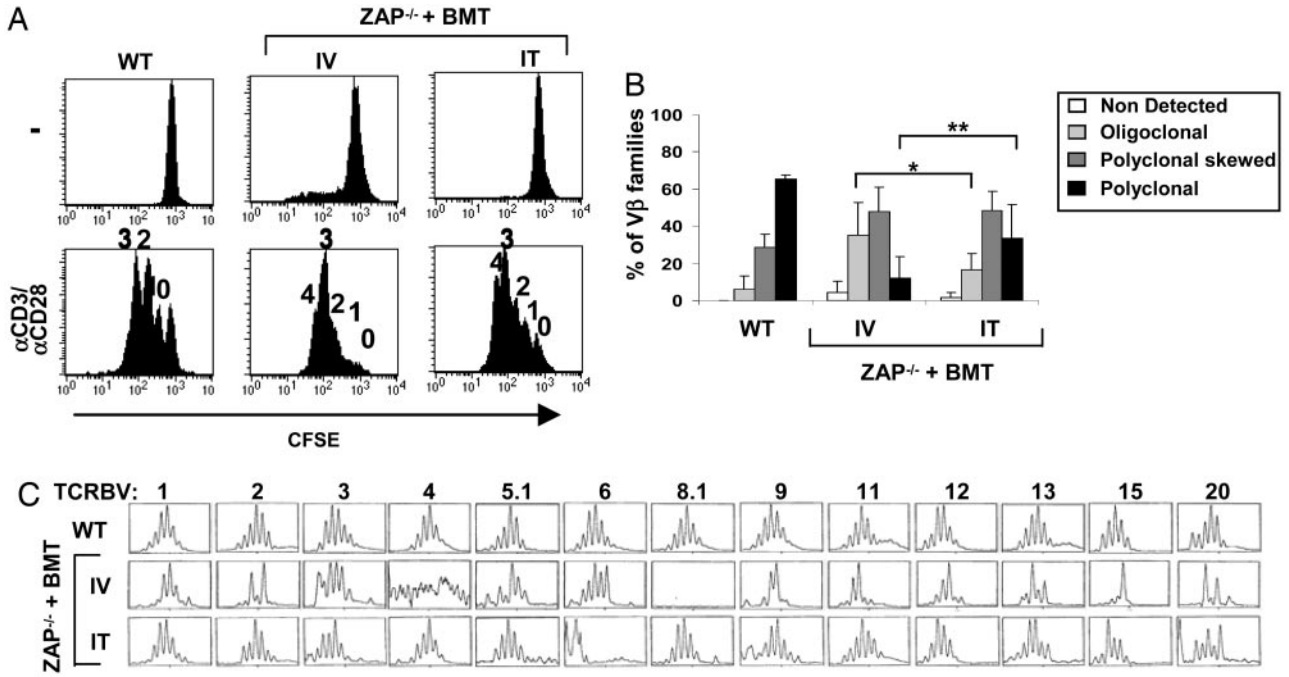

Fig. 3. Function and repertoire diversity of T cells differentiating after IT injection of WT BM progenitors. (*A*) Lymph nodes were recovered from WT mice as well as ZAP$^{-/-}$ mice reconstituted by either i.v. or IT administration of WT BM progenitor cells (2–5 $\times$ 10^5 per mouse). Cells were labeled with the fluorescent dye 5-carboxyfluorescein diacetate succinimidyl ester (CFSE) and cultured *in vitro* in the absence (−) or presence of αCD3 and αCD28 antibodies (1 μg/ml). Three days after activation, CFSE intensity in T lymphocytes was assessed by flow cytometry. The numbers shown above the peaks indicate the number of cell divisions. (*B*) Bar graph quantification of TCR diversity profiles from 22 TCRBV families. Results are expressed as the percentages of nondetected, oligoclonal ($\leq$4 peaks), polyclonal skewed (>4 peaks, shifted CDR3 distribution) or polyclonal (>4 peaks, Gaussian distribution) BV families from WT (n = 3), as well as i.v.-reconstituted (n = 7) and IT-reconstituted (n = 5) ZAP$^{-/-}$ mice. *, $P \leq 0.05$; **, indicates $P \leq 0.01$. (*C*) ZAP$^{-/-}$ mice were injected with lin- WT BM progenitors through i.v. or IT administration. At 12–14 weeks after transplantation, TCRBV repertoires were assessed in peripheral T cells by comparison of TCR CDR3 size distribution obtained after PCR amplification (Immunoscope profiles). Results of 13 representative BV families are shown as density peak histograms with fluorescence intensity in arbitrary units (y axis) plotted against CDR3 size (x axis).

to those receiving 2 $\times$ 10^5 BM cells through i.v. injection. Thus, an $\approx$10-fold lower number of progenitor cells is required to achieve the same level of T cell reconstitution upon their IT administration as compared with i.v. administration.

Discussion

Progress in hematopoietic stem cell transplantation has resulted in a significantly improved outcome for SCID patients (1). However, as T cell differentiation in transplanted SCID patients requires at least 100–150 days and high numbers of injected CD34$^+$ cells (up to 10^7/kg) (3), new therapeutic avenues may improve the treatment of these patients. One strategy is gene therapy, wherein the WT gene is introduced *ex vivo* into autologous HSC from the patient. Importantly, this approach has recently been used to successfully treat multiple patients with adenosine deaminase and γc gene mutations (16–20). Additionally, we recently determined that an *in vivo* gene therapy approach, wherein a ZAP-70-expressing lentiviral vector is introduced directly into the thymus of ZAP-70 KO mice, can result in T cell reconstitution (21). Nevertheless, recent adverse events, with three patients developing lymphoproliferative diseases related to gene transfer (22), indicate the necessity of comparing gene therapy-based strategies with improved cell-based therapies. Here, we explored a potential improvement to cell-based BM transplantation, targeting transplanted donor cells to the thymus.

The vast majority of HSC transplantations in SCID patients are performed during infancy and thymopoiesis is known to be more active in infants as compared with adults. Moreover, it appears that gene therapy for older SCID patients is not successful, likely due to limitations in thymopoeisis (23). As such, and to more closely approach the clinical setting, we compared T cell reconstitution after i.v. and IT administration of BM progenitor cells in 2- to 3-week-old infant ZAP-70$^{-/-}$ mice. This approach, achieved by nonsurgical intervention, resulted in a significantly enhanced T cell reconstitution. Moreover, the T cell reconstitution achieved after IT administration of HSC was of a higher "quality" than that obtained after i.v. administration as judged by the: (*i*) lower number of donor HSC required for T cell differentiation; (*ii*) higher number of peripheral CD3+ T cells; (*iii*) higher proportion of peripheral naïve T cells; and (*iv*) increased TCR diversity. The theoretical implication for a patient would be that his/her immune system is superior because of the ability of newly arising T cells to respond to a larger number of different antigens.

One of the major questions regarding "conventional" i.v. stem cell transplantation for SCID revolves around the issue of whether the engrafted donor cells are progenitor cells or "true" stem cells. Although the long-term presence of peripheral T cells was once taken as an indication of true stem cell engraftment in SCID patients, we now know that peripheral T cells, or their clones, can have a lifespan of >10 years (24). Moreover, data suggesting that donor HSC do not engraft after i.v. administration of HSC into SCID patients are as follows: (*i*) only few naïve T cells are detected 10 years after transplantation, correlating with a skewing of the TCR repertoire (13); (*ii*) the number of T cell receptor excision circles, representing thymic export, decreases to negligible levels within 18 years after transplantation whereas in normal individuals, this process occurs over 80 years (3); and (*iii*) T cell function declines at late time points after transplantation (1). In agreement with this premise, we observed a cessation of thymic differentiation after "conventional" i.v.

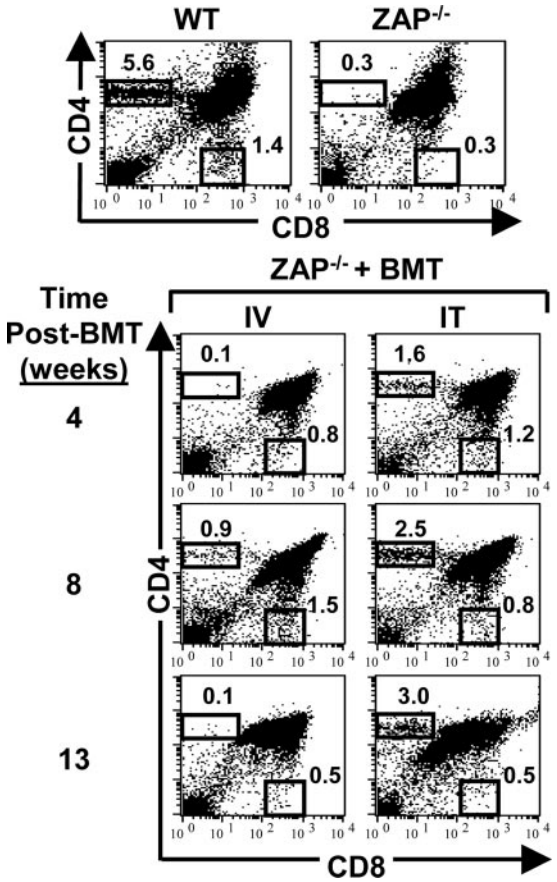

Fig. 4. Kinetics of thymocyte differentiation in ZAP$^{-/-}$ mice after i.v. and IT injection of WT BM progenitor cells. The percentages of SP CD4 and CD8 thymocytes in WT and ZAP$^{-/-}$ mice are shown. Thymocytes were harvested from euthanized ZAP$^{-/-}$-injected mice at different time points after i.v. or IT administration of WT BM cells. At 4, 8, and 13 weeks after BMT, the percentages of SP CD4 and CD8 thymocytes were assessed (n = 3–11 per group).

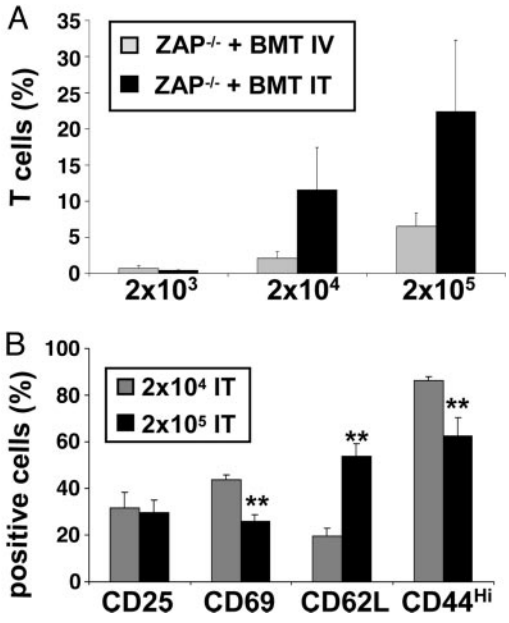

Fig. 5. T cell reconstitution of ZAP-70$^{-/-}$ mice requires 10-fold fewer numbers of BM progenitor cells upon IT injection. (*A*) ZAP$^{-/-}$ mice received 2×10^3, 2×10^4, or 2×10^5 WT *lin*- BM cells by i.v. or IT administration. Lymph node cells were harvested from euthanized animals at 14 weeks after BMT and the percentages of CD3$^+$ T cells were monitored by cytometry. Results are presented as means ± SD (n = 3 per group). (*B*) Bar graph quantification of the percentages of CD3$^+$ T cells expressing CD25, CD69, CD62L, and CD44 markers 14 weeks after IT injection of either 2×10^4 or 2×10^5 WT BM cells. Results are presented as means ± SD (n = 3 per group). **, indicates $P \leq 0.01$.

injection of wild-type BM cells into nonconditioned ZAP-70-deficient mice. Nevertheless, in ZAP-70-deficient mice that were lethally irradiated before transplantation, we previously found that thymocyte differentiation continues for at least 18 weeks after i.v. injection of HSC (25). Moreover, in rat/mice transplantation models, irradiation appears to augment the differentiation of donor thymocytes differentiating from i.v.-administered BM (26–28). Thus, as in humans, stem cell engraftment, at least after their i.v. administration, appears to be modulated by prior myeloablative conditioning.

The capacity of i.v.-injected progenitor cells to enter the thymus in transplanted SCID patients may be modulated by the nature of the mutation. Prockop and Petrie (29) elegantly showed that high numbers of immature double-negative thymoctes, irrespective of the total thymocyte cellularity, result in a resistance of immunodeficient animals to expansion of wild-type T progenitor cells in the thymus. For example, Rag$^{-/-}$ mice, with high numbers of DN cells, but not $\gamma c^{-/-}$ mice, with low numbers of DN thymocytes, are resistant to reconstitution by transplanted WT BM in the absence of conditioning (29). In human SCID patients, it appears that T cell reconstitution occurs as a result of limited waves of differentiation in the thymus, rather than a production of T cells throughout the life of the individual (3, 13). Thus, conventional HSC transplantation may result in progenitor cell entry and expansion in the thymus but at suboptimal levels.

The advantages of IT HSC transplantation are likely due, at least in part, to a more robust thymocyte maturation. Indeed, although many T lymphoid progenitor populations can give rise to T cells when injected into the thymus, most of these cells are not capable of homing to the thymus (14, 15). The BM selection protocol used here, eliminating lineage-differentiated cells, resulted in the injection of multiple cell types identified to have restricted lymphoid progenitor capacity (30, 31). As such, all cells capable of T lineage differentiation could potentially expand when injected directly into the thymus, whereas only the subsets of progenitor cells with thymic-homing capacity (32, 33) could differentiate after their i.v. administration. Similarly, it appears that the differentiation of stem cells in the BM is enhanced when the cells are directly "homed" to the BM through their intrafemoral injection (34, 35).

Differentiating thymocytes have a limited lifespan, i.e., DP cells can differentiate and migrate across the cortex in 15 days (15). As such, a committed thymocyte progenitor is not expected to sustain thymopoiesis over an extended time period. Indeed, under physiological conditions, the thymus does not sustain the maintenance of a progenitor cell with self-renewal capacity (26–28). We were therefore surprised to detect thymocyte differentiation 12–14 weeks after IT administration of wild-type HSC. At least three interpretations are possible: (*i*) the SP thymocytes reflect recirculating mature peripheral T cells; (*ii*) a subset of the IT-injected lin- BM cells, normally incapable of thymic homing, maintains thymopoiesis for an extended time period; or (*iii*) a subset of the IT-injected lin- cells leaves the thymus, reentering during "receptive" periods (5). The first possibility appears unlikely for the following reasons: (*i*) peripheral T cells recirculate with higher frequency in irradiated mice and show higher entry of CD8$^+$ cells (36), whereas our mice were not irradiated and presented with higher percentages of CD4$^+$ SP cells, and (*ii*) at 14 weeks, SP cells were not detected in the

majority of mice transplanted by i.v. administration (9 of 11 mice; Fig. 4) despite the presence of circulating peripheral T cells. With regard to the second hypothesis, the supposition that a BM progenitor cell may sustain thymopoiesis for a longer time period than a thymic progenitor cell is supported by murine data showing that at late time points after transplantation (>28 days), the percentage of donor-derived thymocytes arising from transplanted BM progenitors becomes significantly higher than that arising from thymocyte progenitors (31, 33). Regardless of the precise mechanism(s) involved, our data indicate that IT administration of *lin-* BM progenitors into nonconditioned ZAP-$70^{-/-}$ mice results in a more rapid kinetics of thymocyte differentiation that is sustained for at least 14 weeks.

The ability of IT-injected cells to maintain T cell differentiation over the life of an individual will be crucial to the success of such a strategy. It is thus notable that significantly higher levels of peripheral T cell reconstitution were still detected in IT-reconstituted mice at late time points (20 weeks) after transplantation (unpublished observations). Moreover, previous groups have found that IT-injected progenitor cells have the capacity to differentiate into other hematopoietic lineages, including myeloid, B, and even erythroid cells (37, 38). Indeed, we detected donor B cells (range, 2–4%) in lymph nodes after both i.v. and IT administrations of lin- BM cells. The potential long-term differentiation of diverse hematopoietic lineages after IT injection of progenitor cells remains to be explored.

IT administration of progenitor cells is potentially attractive for patients who meet the following criteria: (*i*) young children where the thymus is not yet involuted; (*ii*) only limiting numbers of donor progenitor cells are available; and (*iii*) mismatched transplants wherein smaller cell doses may lead to reduced graft versus host disease. In the context of SCID, this protocol would be more accessible in the subset of patients with a relatively normal thymus size. In view of a potential clinical application, we recently assessed this approach in macaques by performing nonsurgical IT injection under ultrasound-guided endoscopy (P. Moullier, unpublished observations). After anesthesia of the animals, injections were accomplished within 1 h, indicating that the technical gesture was simple and feasible. Thus, this approach, modulating the administration of allogeneic hematopoietic stem cells, enhances the dynamics of T cell reconstitution and, as such, may improve the outcome of SCID patients. Moreover, IT administration of HSC can be envisioned in patients transplanted for other pathologies, wherein an optimal and rapid T cell reconstitution would be advantageous.

We thank HRH Ph. Moullier for his scientific input and for sharing unpublished data; D. Klatzmann and members of our lab for their scientific critique and support; M. Sitbon for constructive criticisms; and I. Goldschneider, H. Petrie, and A. Singer for helpful input. O.A., R.R.V., and V.S.Z. are supported by fellowships from the French Ministry of Higher Education, Association Française Contre les Myopathies (AFM), and consecutive fellowships from the Fondation pour la Recherche Médicale and Association pour la Recherche sur le Cancer, respectively. This work was supported by grants from the AFM, Immune Deficiency Foundation, Association Franco-Israelienne pour la Recherche Scientifique et Technologie, Centre National de la Recherche Scientifique, and Institut National de la Santé et de la Recherche Médicale.

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